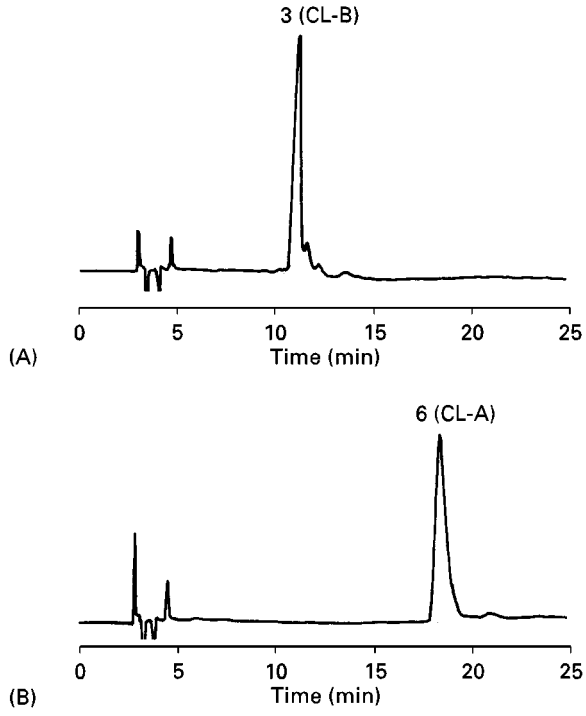

Figure 14 HPLC analysis of CL components of HSCCC fractions. (A) CL-A (Fraction 5); (B) CL-B (Fraction 3). (Reproduced with permission from Oka H *et al.* (1998).)

ideal method for separation and purification of antibiotics.

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent. Chromatography: Instrumentation. **Chromatography: Liquid:** Countercurrent Liquid Chromatography. **III / Antibiotics:** Liquid Chromatography. Supercritical Fluid Chromatography.

Further Reading

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DropBooks

Liquid Chromatography

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Introduction

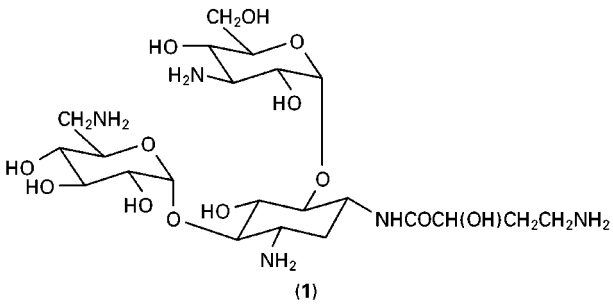
High performance liquid chromatography (HPLC) has been widely used for the analysis of antibiotics because it is superior to conventional microbiological assays in terms of specificity, sensitivity and analysis time. In this article, HPLC conditions for the analysis of a variety of antibiotics are summarized. For analysis of biological samples, not only extraction methods but also derivatization methods are described, if necessary. Since it is not possible to list HPLC methods for all antibiotics in clinical use, only a few have been chosen from each class. Where a stereoisomer exists for the antibiotic of interest, the HPLC conditions that are able to resolve stereoisomers are described.

Aminoglycosides

Aminoglycosides are analysed by reversed-phase HPLC. However, derivatization is usually necessary owing to very poor UV or visible absorption. For detection of aminoglycosides without derivatization, electrochemical, refractive index or mass spectrometric detection may be used.

Amikacin

For determination of amikacin (**Figure 1**, structure 1), the serum sample is loaded onto the silica gel column, followed by addition of *o*-phthalaldehyde (a derivatizing reagent). The column is eluted with 95% ethanol (pH 10) and the eluent is heated at 50°C. After cooling, the mixture is injected onto an ODS



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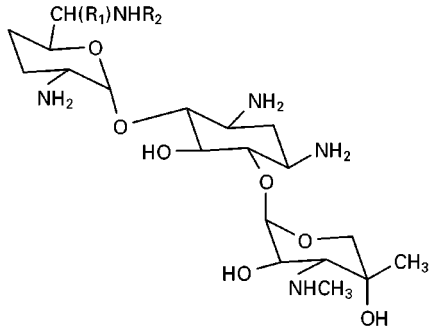


	R_1	R_2
2	CH_3	CH_3
3	H	H
4	CH_3	H

Figure 1 Chemical structures of amikacin (1) and gentamycins C_1 (2), C_{1a} (3) and C_2 (4).

column. Mobile phase is methanol–water–acetonitrile (65 : 30 : 5) containing 0.2% tripotassium ethylenediamine tetraacetic acid (EDTA). Amikacin is detected fluorometrically at 350 nm for excitation and 420 nm for emission. The detection limit is 1 μg mL^{-1}. In order to resolve amikacin from its three isomers, a similar method is used except that the mobile phase is methanol–water (7 : 3). Tobramycin may be analysed with the same method.

Pre-column derivatization of amikacin is also conducted by addition of 1-fluoro-2,4-dinitrobenzene to plasma or urine, leading to formation of a stable chromophore, which can be detected at 360 nm. An ODS column is used with a mobile phase consisting of acetonitrile–water (68 : 32). The detection limit is 1 μg mL^{-1}. Amikacin may be extracted from serum using a cation exchange solid-phase extraction column prior to derivatization.

Gentamycin

Gentamycin in plasma is extracted with a cation exchange solid-phase extraction column (carboxypropyl-bonded silica column). Gentamycin is eluted with a 1 : 1 mixture of acetonitrile–0.2 mol L^{-1} borate buffer (pH 10.5) and is derivatized with 9-fluorenylmethyl chloroformate. Derivatized gentamycin is analysed on an ODS column with a mobile phase consisting of acetonitrile–water (9 : 1). The derivatives are detected fluorometrically at 260 nm excitation, 315 nm emission, with a detection limit of less than 50 ng mL^{-1}. Gentamycins C_1, C_{1a} and C_2 are resolved (Figure 1, structures 2, 3 and 4, respectively).

o-Phthaldialdehyde, dansyl chloride, fluorescamine, 1-fluoro-2,4-dinitrobenzene or 2,4,6-trinitrobenzenesulfonic acid may also be used as derivatizing reagents.

Glycopeptides

Various glycopeptide antibiotics are separated with an ODS column. The mobile phase composition is either 7–32% acetonitrile (7% for 1 min, then increase to 34% over 13 min) in 0.1 mol L^{-1} phosphate buffer (pH 3.2) or 5–35% acetonitrile (5% for 1 min, then increase to 35% over 13 min) in 0.025 mol L^{-1} phosphate buffer (pH 6.0). Glycopeptides are detected at 220 nm.

Vancomycin

Serum is deproteinized with an ice-cold mixture of 10% trichloroacetic acid-acetone (2 : 1) and the supernatant is injected into an ODS column. The mobile phase consists of 50 mmol L^{-1} sodium dihydrogen phosphate (pH 3.3)–acetonitrile (4 : 1) containing 1 mmol L^{-1} sodium dodecyl sulfate. Vancomycin is detected at 235 nm with a detection limit of 1 μg mL^{-1}.

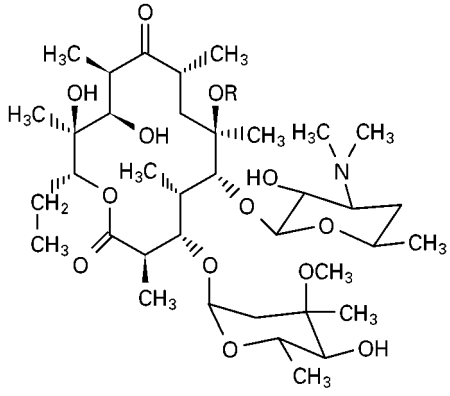
Macrolides

Clarithromycin

Clarithromycin (**Figure 2**, structure 5) and its major metabolite (14-hydroxyclarithromycin) are analysed with a C_8 column. The mobile phase consists of acetonitrile–methanol–water (39 : 9 : 52) containing 0.04 mol L^{-1} sodium dihydrogen phosphate with the pH being adjusted to 6.8 using sodium hydroxide. The eluent is monitored by electrochemical detection with a quantification limit of 30 ng mL^{-1}. Plasma and urine are extracted with ethyl acetate–hexane (1 : 1).

Erythromycin

Erythromycin A (the major and most active component, **Figure 2**, structure 6), erythromycin B,



	R
5	CH_3
6	H

Figure 2 Chemical structures of clarithromycin (5) and erythromycin A (6).

erythromycin C and related compounds in commercial preparations are analysed with an ODS column using a mobile phase consisting of acetonitrile–methanol–0.2 mol L^{-1} ammonium acetate–water (45 : 10 : 10 : 35, pH adjusted to 7.0–7.8). Erythromycins are detected at 215 nm.

Erythromycin and its metabolites in biological fluids are analysed with an ODS column using a mobile phase consisting of acetonitrile–methanol–0.2 mol L^{-1} sodium acetate (pH 6.7, 40 : 5 : 55). Erythromycin is detected with a dual-electrode electrochemical detector with a detection limit of 10 ng mL^{-1} in plasma. Erythromycin is extracted from plasma with ether, and urine is deproteinized with acetonitrile. Other related erythromycins and degradation products are also resolved.

Ivermectin

Ivermectin in tissue is analysed with an ODS column using a mobile phase of acetonitrile–water (9 : 1) at 65°C. Ivermectin is detected fluorometrically at 272 nm excitation, 465 nm emission, with a detection limit of 0.25 ng g^{-1}. Tissue sample is loaded onto a C_8 solid-phase extraction column, eluted with acetonitrile, and the eluate dried under a stream of N_2. The dried residue is dissolved with ethy1 acetate–hexane (2 : 3), loaded on a silica column, and eluted with methanol–ethyl acetate (1 : 1). The eluate is dried under a stream of N_2 and treated with trifluoroacetic anhydride and methylimidazole. The analyte thus obtained is injected into an HPLC.

Penicillins and Cephalosporins

Many penicillins and cephalosporins are chiral, partly due to the chirality of the side chain. The D-epimers of ampicillin (see **Figure 3**, structure 13), cephalexin (**Figure 3**, structure 17) and cephaloglycin are more active than the corresponding L-epimers. Stereoisomers also exist for amoxicillin (**Figure 3**, structure 14), azidocillin, cefamandole, cefsulodin and ceftibuten (**Figure 3**, structure 16). For these β-lactams, commercial preparations contain only a single isomer.

For some β-lactams, commercial preparations contain both epimers. These include carbenicillin (7), clometocillin, moxalactam (18), phenethicillin (11), propicillin (12), sulbenicillin (8), temocillin (9) and ticarcillin (10) (see **Figure 3**). Epimers of these β-lactams are resolved by reversed-phase HPLC.

Ampicillin

In order to separate ampicillin (**Figure 3**, structure 13) from penicilloic acid, phenylglycine and 6-aminopenicillanic acid, an ODS column is used with a mobile phase consisting of 35% acetonitrile in an aqueous solution of 3.5 mmol L^{-1} sodium dodecyl sulfate and 0.2 mol L^{-1} formic acid. Ampicillin and other compounds are detected at 254 nm. For separation of ampicillin from its degradation products, an ODS column is used with a mobile phase of 22.5% methanol in an aqueous solution of 5 mmol L^{-1} tetrabutylammonium hydrogen sulfate and 5 mmol L^{-1} ammonium sulfate (pH 2.6). Ampicillin and the degradation products are detected at 238 nm.

Ampicillin is analysed in biological fluids with an ODS column using a mobile phase consisting of 0.06 mol L^{-1} phosphate buffer (pH 4.6)–methanol (425 : 75). Ampicillin is detected at 225 nm with a limit of accurate determination of 0.5 μg mL^{-1} in urine, plasma or saliva. Samples are deproteinized with perchloric acid.

In order to increase sensitivity, ampicillin and its metabolites in urine are subjected to postcolumn alkaline degradation following separation with an ODS column. Urine is diluted with water and injected directly. The mobile phase is an aqueous mixture of 5 mmol L^{-1} sodium heptylsulfonate, 1 mmol L^{-1} sodium dihydrogen phosphate and 9 mmol L^{-1} phosphoric acid–methanol (1.5 : 1, pH 3.0). Degradation of ampicillin and its metabolites is conducted with 0.75 mol L^{-1} sodium hydroxide, 2 mmol L^{-1} mercuric chloride and 10 mmol L^{-1} EDTA, and the degradation products are detected at 300 nm. The limits of accurate determination are 0.5 μg mL^{-1} for ampicillin and 1–2 μg mL^{-1} for the metabolites.

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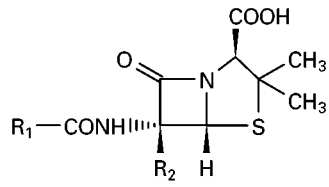

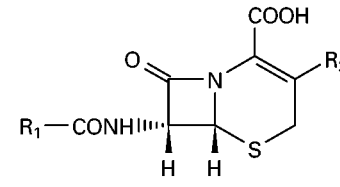

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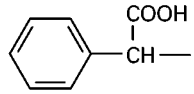
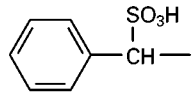
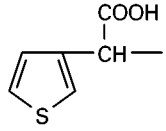
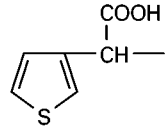
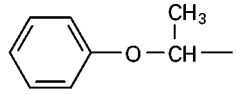
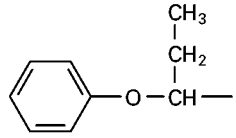
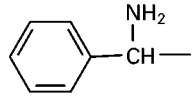
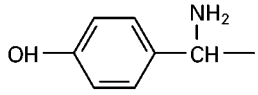
7		H
8		H
9		OCH_3
10		H
11		H
12		H
13		H
14		H

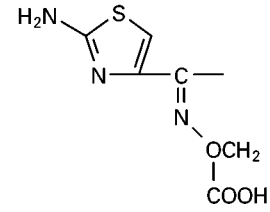
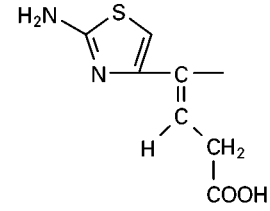
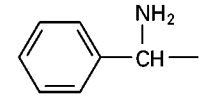
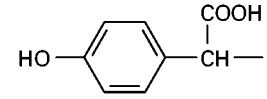
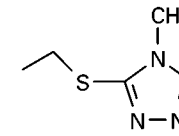
	R_1	R_2
15		$-CH=CH_2$
16		H
17		CH_3
18		

Figure 3 Chemical structures of carbenicillin (7), sulbenicillin (8), temocillin (9), ticarcillin (10), phenethicillin (11), propicillin (12), ampicillin (13), amoxicillin (14), cefixime (15), ceftibuten (16), cephalexin (17) and moxalactam (18).

Carbenicillin, Sulbenicillin and Ticarcillin

These epimeric, di-anionic β-lactams are similar in physicochemical properties and are analysed under very similar conditions. For analysis of carbenicillin (**Figure 3**, structure 7), plasma and urine samples are loaded onto an anion exchange solid-phase extraction column. Carbenicillin epimers are eluted with 10% lithium chloride-methanol (3 : 2) and injected into an HPLC. Analysis is on an ODS column with a mobile phase consisting of 0.05 mol L^{-1} ammonium acetate–methanol (9 : 1). Carbenicillin

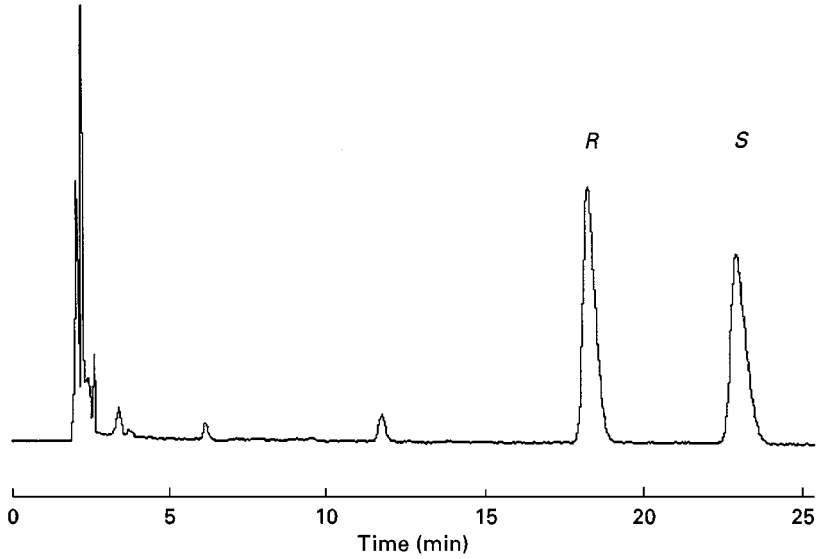


Figure 4 Chromatogram of human plasma spiked with carbenicillin. Five hundred microlitres of human plasma was spiked with 20 μL of an aqueous solution of carbenicillin (5.2 mg mL^{-1}). *R*, *R*-epimer, *S*, *S*-epimer.

epimers are detected at 254 nm. The epimers are resolved to the baseline with the *R*-epimer being eluted prior to the *S*-epimer (**Figure 4**).

Similar methods can be applied for the analysis of sulbenicillin (**Figure 3**, structure 8) and ticarcillin (**Figure 3**, structure 10). For sulbenicillin, the mobile phase consists of 0.05 mol L^{-1} phosphate buffer (pH 7.0)–methanol (8 : 1). The epimers are resolved to the baseline with the *S*-epimer being eluted faster than the *R*-epimer. The detection limit is 0.5 μg mL^{-1} for each epimer. For ticarcillin, the mobile phase consists of 0.05 mol L^{-1} phosphate buffer (pH 7.0)–methanol (12 : 1). The epimers are resolved to the baseline with the *R*-epimer being eluted faster than the *S*-epimer.

Cefixime

Cefixime (**Figure 3**, structure 15) is determined on an ODS column with a mobile phase consisting of acetonitrile–water (2.75 : 7.25) containing 0.01 mol L^{-1} ammonium acetate and 0.01 mol L^{-1} tetra-*N*-butylammonium bromide. Cefixime is detected at 290 nm.

For analysis of cefixime in serum, an ODS column is used with a mobile phase consisting of 0.3% potassium dihydrogen phosphate–acetonitrile (88.5 : 11.5). For urine analysis, the mobile phase is a mixture of 0.15% potassium dihydrogen phosphate–acetonitrile (77 : 23) containing 0.1% phosphoric acid. Cefixime is detected at 254 nm with a detection limit of 0.1 μg mL^{-1}.

Ceftibuten

Although commercially available formulations contain only the *cis* isomer (**Figure 3**, structure 16), HPLC methods for determination of both *cis* and *trans* isomers have been developed because isomerization is observed *in vivo*.

Ceftibuten and its *trans* isomer are separated with an ODS column using a mobile phase consisting of 100 mmol L^{-1} ammonium acetate–methanol (92 : 8). Both isomers are detected at 262 nm.

An HPLC method for ceftibuten isomers is also developed for plasma and urine samples. Samples are deproteinized with ethanol and injected into an ODS column with a mobile phase composed of PIC A (tetrabutylammoniumphosphate)–acetonitrile–methanol (50 : 6 : 3). Both isomers are detected at 256 nm with a detection limit of 1 μg mL^{-1} for each isomer.

Cephalexin

Cephalexin epimers are separated with an ODS column using a mobile phase of 0.1 mol L^{-1} phosphate buffer (pH 3.5)–methanol (95 : 5). The epimers are detected at 254 nm. The two epimers are separated to the baseline with the L-epimer being eluted prior to the D-epimer.

Cephalexin epimers in serum and urine are analysed using a TSK-gel ODS-80 TM column after deproteinization with methanol. Mobile phase compositions are 10 mmol L^{-1} ammonium acetate–methanol (4 : 1) for determination of the D-epimer, and 10 mmol L^{-1} phosphate buffer (pH 3.0)–methanol (9 : 1) containing 10 mmol L^{-1} ammonium acetate and 10 mmol L^{-1} pentanesulfonic acid for determination of the L-epimer. The epimers are detected at 260 nm.

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Other β-Lactams

Epimers of phenethicillin (PEPC, **Figure 3**, structure 11), propicillin (PPPC, **Figure 3**, structure 12) and clometocillin are analysed with a Zorbax C_8 column. The mobile phase is composed of methanol–water–5% 0.2 mol L^{-1} phosphate buffer (pH 7.0) and the epimers are detected at 254 nm. Ratios of methanol in the mobile phase are 37.5, 45 and 50% for PEPC, PPPC and clometocillin, respectively. Epimers are resolved close to the baseline, and the D-epimers elute faster than the corresponding L-epimers.

The same HPLC conditions can be used for the analysis of ampicillin, amoxicillin and azidocillin, except that the methanol content is varied between 10 and 40%. The L-epimer elutes faster for ampicillin, whereas the D-epimer elutes faster for amoxicillin and azidocillin. The less active epimers are not detected in the commercial preparations of these penicillins.

Epimers of PEPC and PPPC are also resolved with an ODS column. The mobile phase consists of 100 mmol L^{-1} ammonium acetate–methanol (62 : 38 and 58 : 42 for PEPC and PPPC, respectively) with a UV detection at 220 nm. PEPC and PPPC epimers are baseline separated (**Figure 5**).

Bacampicillin and cefotiam hexetil are the prodrugs of ampicillin and cefotiam, respectively, which are commercially available as mixtures of two epimers due to chirality of the prodrug moiety. For separation of bacampicillin isomers, an ODS column is used with a mobile phase consisting of 20 mmol L^{-1} ammonium acetate–methanol (45 : 55). The isomers are detected at 220 nm. For separation of the isomers of cefotiam hexetil, an ODS column is used with a mobile phase consisting of 50 mmol L^{-1} phosphate buffer (pH 3.0)–acetonitrile (73 : 27). The isomers are detected at 262 nm. Baseline separation of the isomers of bacampicillin and cefotiam hexetil are observed.

Semisynthetic cephalosporins are extracted from biological fluids and chromatographed with an ODS column. Urine samples are merely centrifuged and diluted with distilled water. Serum samples are mixed with 0.4 mol L^{-1} HCl and extracted with $CHCl_3$-*n*-pentanol (3 : 1). The organic phase is re-extracted into phosphate buffer (pH 7), which is injected into the HPLC. The mobile phase is 0.01 mol L^{-1} acetate buffer (pH 4.8)–methanol (15 : 85) with detection wavelengths of 254, 245, 234, 275, 270, 240 and 240 nm for cefuroxime, cefoxitin, cefotaxime, cefazolin, cefamandole, cephalotin and cefoperazone, respectively.

Cephalosporins in serum are also analysed with an octyl column using a mobile phase of methanol–12.5 mmol L^{-1} phosphate buffer (pH 2.6, 1 : 4). Cefaclor, cefadroxil, cefixime, cephalexin and cephradine are simultaneously analysed and detected at 240 nm. The detection limits are 0.1 μg mL^{-1} for cefixime and 1.0 μg mL^{-1} for other cephalosporins. Serum is deproteinized with acetonitrile.

Cephalosporins with a tetrazole ring are analysed from plasma with an ODS column using a mobile phase consisting of 0.05 mol L^{-1} phosphate buffer (pH 6.6)–methanol with ratios of 3 : 1 and 2 : 1 for cefamandole and cefoperazone, respectively. For cefotiam and cefmetazole, a mixture of phosphate buffer-tetrahydrofuran (20 : 1) is used as a mobile phase. Cephalosporins are detected at 254 nm with a limit of detection of 1 μg mL^{-1} for all cephalosporins.

In order to increase sensitivity, ampicillin, amoxicillin, cephalexin and cephradine in plasma are assayed after formation of fluorescent degradation products. Plasma is deproteinized with 10% trichloroacetic acid and the supernatant is heated under various conditions to form degradation products. The degradation products are extracted with an organic solvent and injected into a Nucleosil C_{18} column at

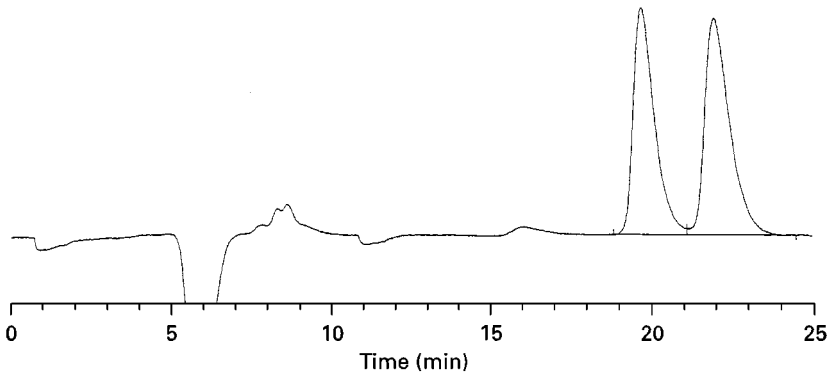

Figure 5 Chromatogram of phenethicillin. One hundred microlitres of an aqueous solution of phenethicillin (22 μg mL^{-1}) was directly injected onto HPLC.

Table 1 HPLC conditions for β-lactams

β-Lactam	*Stationary phase*	*Mobile phase*	*Detection*
Benzylpenicillin	ODS	Methanol–0.05 mol L^{-1} ammonium carbonate (1 : 3)	UV (254 nm)
Benzylpenicillin	ODS	Phosphate buffer (pH 6.0)–acetonitrile (4 : 1)	UV (225 nm)
Cefsulodin	ODS	16.8 mmol L^{-1} dibasic ammonium phosphate–acetic acid–methanol (100 : 1.68 : 5.98) containing 5 mmol L^{-1} triethylamine	UV (260 nm)
Cefsulodin	ODS	Aqueous solution (containing 38.8 mmol L^{-1} ammonium acetate, 0.292 mmol L^{-1} dibasic ammonium phosphate and 9.363 mmol L^{-1} triethylamine)–acetonitrile–methanol–dimethylformamide–acetic acid (1000 : 7.06 : 1.05 : 1.31 : 0.30)	UV (260 nm)
Moxalactam	ODS	Methanol–0.05 mol L^{-1} monobasic potassium phosphate (5 : 95) adjusted to pH 6.5	UV (254 nm)
Moxalactam	ODS	Methanol–0.005 mol L^{-1} tetra-*n*-butylammonium phosphate (1 : 3) adjusted to pH 6.0	UV (254 nm)
Moxalactam	ODS	0.1 mol L^{-1} Ammonium acetate–acetonitrile (95 : 5) adjusted to pH 6.5	UV (270 nm)
Moxalactam	ODS	0.1 mol L^{-1} Sodium phosphate–methanol (84 : 16) adjusted to pH 3.2	UV (254 nm)
Temocillin	ODS	Methanol-0.1 mol L^{-1} phosphate buffer (pH 7.0, 1 : 9)	UV (230 nm)
Temocillin	Octyl silane	Methanol–0.1 mol L^{-1} phosphate buffer (pH 7.0, 16 : 84)	UV (230 nm)
7-Ureidoacetamido cephalosporins	ODS	0.01 mol L^{-1} Diammonium hydrogen phosphate containing 5–20% methanol	UV (254 nm)

55°C. The mobile phase consists of methanol–water (3 : 2) with a fluorescent detection at 345 nm (excitation) and 420 nm (emission) for ampicillin, cephalexin and cephradine. For amoxicillin, the mobile phase is methanol–water (55 : 45) with a fluorescent detection at 355 nm (excitation) and 435 nm (emission). Detection limits are 0.5 ng mL^{-1} for ampicillin, 2 ng mL^{-1} for cephalexin and 10 ng mL^{-1} for amoxicillin and cephradine. For sensitive determination of β-lactams, pre-column derivatization with imidazole–metal salt reagent or formaldehyde, or post-column derivatization with *o*-phthaldialdehyde or fluorescamine may be applied.

HPLC conditions for several other β-lactams are summarized in **Table 1**. Epimers of these β-lactams are separated using the conditions listed in Table 1, except for benzylpenicillin.

Fluoroquinolones

Among fluoroquinolone antibiotics, lomefloxacin, ofloxacin and temafloxacin are used clinically as the racemates (see **Figure 6**, structures 19, 20 and 21, respectively). Therefore, enantiospecific HPLC methods are described below for these fluoroquinolones. Non-chiral HPLC conditions for the chiral as well as other non-chiral fluoroquinolones are summarized in **Table 2**. Detection limits listed in **Table 2** are mostly those for plasma or serum analysis.

Lomefloxacin

Lomefloxacin enantiomers are extracted from plasma at pH 7 with a mixture of chloroform–isopentyl alcohol–diethyl ether (71.25 : 3.75 : 25) and derivatized with *S*-(+)-(1-naphthyl)ethylisocyanate to form diastereomers. Derivatized diastereomers are analysed with a Radial Pak normal-phase column using a mobile phase of hexane–chloroform–methanol (64.5 : 33 : 2.5). Diastereomers are detected fluorometrically at 280 and 470 nm for excitation and emission. The limit of accurate quantification is less than 10 ng mL^{-1} for each enantiomer.

Ofloxacin

Serum and urine samples are diluted with 0.1 mol L^{-1} phosphate buffer (pH 7.0) and extracted with dichloromethane. Ofloxacin enantiomers in the extract are reacted with L-leucinamide to form diastereomers. The diastereomers are extracted with 1 mol L^{-1} HCl, and injected into an ODS column. The mobile phase is 0.2 mol L^{-1} phosphoric acid (with the pH adjusted to 1.85 with tetraethylammonium hydroxide)–acetonitrile (4 : 1) with fluorescence detection at 298 nm excitation and 458 nm emission. The derivative of the *S*-(−)-enantiomer elutes prior to that of the *R*-(+)-enantiomer with baseline separation. Detection limits are 3 and 80 ng mL^{-1} for plasma and urine, respectively.

Ofloxacin enantiomers are also analysed using a chiral stationary phase (bovine serum albumin

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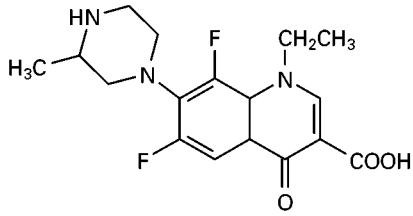

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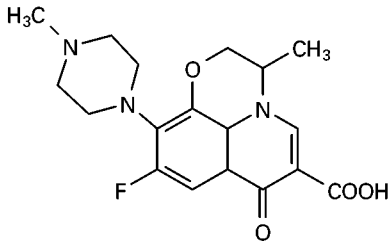

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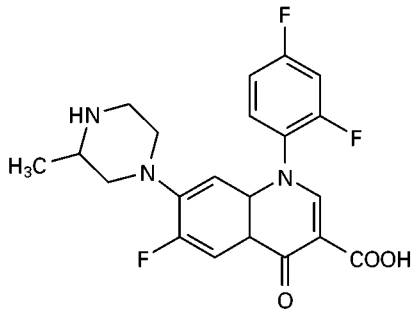

(21)

Figure 6 Chemical structures of lomefloxacin (19), ofloxacin (20) and temafloxacin (21).

covalently bonded to silica) without derivatization. Mobile phase is 0.2 mol L^{-1} phosphate buffer (pH 8.0)–2-propanol (97 : 3). Enantiomers are detected fluorometrically at 298 nm excitation, 458 nm emission. Resolution and sensitivity are poorer than those for the above derivatization method.

For clinical use, ofloxacin has been changed to levofloxacin which is the pharmacologically active *S*-(−)-enantiomer.

Temafloxacin

Temafloxacin enantiomers in biological fluids are extracted with methylene chloride and analysed by two types of HPLC method with derivatization. For the first method, temafloxacin enantiomers are reacted with *S*-(−)-*N*-1-(2-naphthylsulfonyl)-2-pyrrolidine carbonylchloride to form diastereomers, which are injected into a silica gel column. The mobile phase is hexane–methyl acetate–methanol–ammonia water (150 : 100 : 10 : 1) with UV detection at 280 nm. The detection limit is 5 ng mL^{-1} for each diastereomer with a separation coefficient of 1.05.

For the second method, temafloxacin is reacted with acetic anhydride to form acetylated temafloxacin followed by reaction with isobutylchloroformate to form cabonylamidated derivatives. These double-derivatized temafloxacin enantiomers are analysed with an ovomucoid conjugated silica gel column using a mobile phase of 0.02 mol L^{-1} phosphate buffer (pH 7.0)–acetonitrile (92 : 8). The enantiomers are detected at 280 nm. The detection limit is 5 ng mL^{-1} for each enantiomer with a separation coefficient of 1.50, indicating a better resolution by the second method.

Sulfonamides

Various sulfonamides are analysed with an ODS column using a mobile phase composed of acetic acid–triethylamine–water–acetonitrile–methanol (0.4 : 0.2 : 710 : 100 : 100) and detection at 254 nm. Sulfonamides in formulations are extracted or dissolved using dimethylformamide, methanol or the mobile phase.

Sulfonamides in body fluids are analysed with an ODS column using a mobile phase consisting of acetonitrile-water (1 : 9, changing to 9 : 1 in 10 min). Detection is either UV at 254 nm or with a mass spectrometer. Sulfonamides are extracted with hexane–dichloromethane–ether (1 : 1 : 1) at pH 3.0–3.2.

Sulfamethoxazole

Sulfamethoxazole and its acetylated metabolites in body fluids are analysed with an ODS column using a mobile phase consisting of methanol–1% acetic acid (1 : 4, pH 2.9). Sulfamethoxazole and the metabolites are detected at 230 nm with a detection limit of less than 1 μg mL^{-1}. Plasma is extracted with ethyl acetate, and urine is deproteinized with acetonitrile.

Sulfamethoxazole in body fluids are also analysed with an ODS column using a mobile phase consisting of 0.067 mol L^{-1} phosphate buffer (pH 6.7)–methanol (5 : 1). Sulfamethoxazole is detected at 260 nm with a detection limit of 0.5 μg mL^{-1}.

Sulfasalazine

Sulfasalazine is decomposed in the colon to generate two biologically active drugs, i.e. sulfapyridine and 5-aminosalicylic acid. Sulfasalazine in commercial preparations is analysed with an ODS column using a mobile phase consisting of 10–15% 2-propanol in 0.01 mol L^{-1} phosphate buffer (pH 7.7), and detected at 254 nm. A silica column is also used for analysis of sulfasalazine and its degradation products in commercial preparations with a mobile phase of chloroform–acetonitrile–*n*-butanol (4 : 1 : 1).

Table 2 Non-stereospecific HPLC conditions for chiral as well as non-chiral fluoroquinolones

Fluoroquinolone	*Stationary phase*	*Mobile phase*	*Detection*[1]	*Detection limit*
Enoxacin	ODS	30% Methanol in an aqueous solution of 0.05 mol L^{-1} potassium dihydrogen phosphate and 2% acetic acid	UV (265 nm)	3 pmol
Ciprofloxacin	ODS	An aqueous solution of 18 mmol L^{-1} potassium dihydrogen phosphate and 0.13 mmol L^{-1} heptane sulfonic acid–methanol–phosphoric acid (7 : 3 : 0.01)	Fluorescence (ex. 278 nm, em. 475 nm)	200 ng mL^{-1}
Lomefloxacin	ODS	An aqueous solution of 0.2% sodium acetate trihydrate, 0.2% citric acid monohydrate and 0.1% triethylamine (pH 4.8)–acetonitrile (80 : 23)	Fluorescence (ex. 280 nm, em. 430 nm)	50 ng mL^{-1}
Nalidixic acid	ODS	Water–methanol–cetrimonium bromide (50 : 50 : 0.12)	(UV 313 nm)	1 μg mL^{-1}
Nalidixic acid	Amino-cyano	Methanol–0.1 mol L^{-1} citrate buffer (pH 3, 95 : 15)	(UV 254 nm)	0.1 μg mL^{-1}
Norfloxacin	Anion-exchange	0.05 mol L^{-1} phosphate buffer (pH 7)–acetonitrile (4 : 1)	(UV 273 nm)	0.1 μg mL^{-1}
Norfloxacin	ODS	Acetonitrile–0.01 mol L^{-1} phosphate buffer (pH 2.5) containing 1 mmol L^{-1} triethylamine (11 : 89)	(UV 279 nm)	20 ng mL^{-1}
Ofloxacin	ODS	0.5% Sodium acetate (pH 2.5)–acetonitrile (87 : 13)	(UV 300 nm)	100 ng g^{-1} tissue
Ofloxacin	ODS	Tetrahydrofuran–0.06 mol L^{-1} phosphate buffer (pH 2.6) containing 3% triethylamine (5.5 : 94.5)	Fluorescence (ex. 282 nm, em. 450 nm)	20 ng mL^{-1}
Pefloxacin	ODS	An aqueous solution of 0.2% sodium acetate, 0.2% citric acid and 0.1% triethylamine-acetonitrile (86 : 14)	Fluorescence (ex. 330 nm, em. 440 nm)	50 ng mL^{-1}
Sparfloxacin	ODS	5% Acetic acid–acetonitrile–methanol (70 : 15 : 15)	UV 364 nm	5 ng mL^{-1}
Temafloxacin	ODS	53% Acetonitrile in an aqueous solution of 40 mmol L^{-1} phosphoric acid, 10 mmol L^{-1} sodium dihydrogen phosphate, 0.2% sodium dodecyl sulfate and 5 mmol L^{-1} *N*-acetylhydroxamic acid	Fluorescence (ex. 280 nm, em. 389 nm)	10 ng mL^{-1}
Temafloxacin	ODS	19% Acetonitrile in an aqueous solution of 5 mmol L^{-1} tetrabutylammonium bromide, 10 mmol L^{-1} sodium hydrogen phosphate	Fluorescence (ex. 275 nm, em. 450 nm)	10 ng mL^{-1}
Fleroxacin	ODS	10% Acetonitrile in an aqueous solution of 5 mmol L^{-1} tetrabutylammonium bromide, 10 mmol L^{-1} sodium hydrogen phosphate	Fluorescence (ex. 277 nm, em. 445 nm)	2.5 ng mL^{-1}
Fleroxacin	ODS	Phosphate buffer (pH 3)–acetonitrile–methanol (85 : 7.5 : 7.5) containing tetrabutylammonium hydroxide and triethylamine	Fluorescence (ex. 290 nm, em. 470 nm)	5 ng mL^{-1}

[1]ex., excitation; em., emission.

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Sulfasalazine in plasma is extracted with isoamyl acetate and analysed with an ODS column using a mobile phase of 0.01 mol L^{-1} phosphate buffer (pH 7.7)–acetonitrile (83 : 17). Sulfasalazine is detected at 365 nm with a limit of detection of 5 ng.

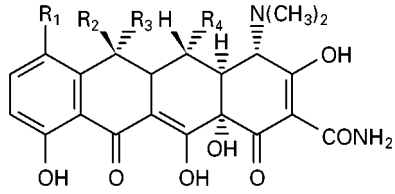
Sulfasalazine, sulfapyridine, 5-aminosalicylate and their metabolites in plasma are analysed with a methylsilane column using a mobile phase of methanol–0.05 mol L^{-1} phosphate buffer (pH 7.4) containing 0.1% tetrabutylammonium hydrogen sulfate (22.5 : 77.5). The eluants are monitored fluorometrically at 320 nm excitation, 389 nm emission, with a detection limit of 0.5 μg mL^{-1}. Plasma is deproteinized with methanol.

Tetracyclines

Various tetracyclines are analysed with an octyl column using a mobile phase of methanol–acetonitrile–0.01 mol L^{-1} aqueous oxalic acid solution (pH adjusted to 2.0 with 28% aqueous ammonia, 1 : 1.5 : 5) and detection at 360 nm.

Tetracycline (TC), chlortetracycline (CTC), doxycycline, minocycline, oxytetracycline (OTC), impurities of these tetracyclines including 4-epitetracycline, anhydrotetracycline and 4-epianhydrotetracycline (a nephrotoxic degradation product) are resolved with an ODS column using a gradient system (see **Figure 7** for chemical structures). The mobile phase is an aqueous solution of 1 mmol L^{-1} tetraammonium ethylenediamine tetraacetate and 50 mmol L^{-1} diethanolamine (pH adjusted to 7.3 with 85% phosphoric acid) containing 2–10% isopropanol. Tetracyclines are detected at 254 nm. Impurities of tetracycline are also analysed with an ODS column using a mobile phase consisting of methanol–acetonitrile–0.2 mol L^{-1} aqueous oxalic acid solution (pH adjusted to 2.0 with 28% aqueous ammonia, 1 : 1 : 3.5). Tetracycline and impurities are detected at 400 nm.

TC, CTC and OTC in plasma and urine are analysed with an ODS column using a mobile phase consisting of 0.01 mol L^{-1} phosphate buffer (pH 2.4)–acetonitrile (7 : 3 or 6 : 4). Tetracyclines are detected at 355 nm with a detection limit of 1 μg mL^{-1}. Extraction of tetracyclines from biological fluids into ethyl acetate is improved by formation of phenylbutazone–tetracycline ion-pairs.



	R_1	R_2	R_3	R_4
22	H	OH	CH_3	H
23	Cl	OH	CH_3	H
24	H	H	CH_3	OH
25	$N(CH_3)_2$	H	H	H
26	H	OH	CH_3	OH

Figure 7 Chemical structures of tetracycline (22), chlortetracycline (23), doxycyline (24), minocycline (25) and oxytetracycline (26).

Other Antibiotics: Azole Antifungals

Itraconazole and its active metabolite (hydroxyitraconazole) in serum are analysed with a Lichrospher RP8 column using a mobile phase of acetonitrile–water (62 : 38) containing 0.05% diethylamine. The pH of the mobile phase is adjusted to 6.0 with 30% acetic acid. Itraconazole and hydroxyitraconazole are detected at 258 nm with detection limits of 10 and 7 ng mL^{-1}, respectively. Serum is extracted with heptane-isoamyl-alcohol (9 : 1).

Itraconazole and hydroxyitraconazole in plasma and tissue are also analysed with an ODS column using a mobile phase of water–acetonitrile–diethylamine (42 : 58 : 0.05). The pH of the mobile phase is adjusted to 2.45 with 85% phosphoric acid. Itraconazole and hydroxyitraconazole are detected fluorometrically at 260 nm excitation and 365 nm emission. Detection limits of itraconazole are 5 ng mg^{-1} and 5 ng mL^{-1} for tissue biopsy and plasma, respectively. Itraconazole in tissue or plasma is extracted with methanol.

Fluconazole in plasma is analysed with an octyl column using a mobile phase of water–acetonitrile (72 : 28). Fluconazole is detected at 260 nm with a detection limit of 0.4 μg mL^{-1}. Plasma is deproteinized with acetonitrile.

Miconazole in plasma is analysed with an ODS column using a mobile phase of methanol–acetonitrile–0.01 mol L^{-1} phosphate buffer (pH 7.0, 36 : 36 : 28). Miconazole is detected at 230 nm with a detection limit of 5 ng mL^{-1}. Plasma is treated with an octadecyl solid-phase extraction column prior to HPLC analysis.

Econazole in serum is determined with an ODS column using a mobile phase of 0.01 mol L^{-1} potassium dihydrogen phosphate–methanol (3 : 7), with the pH being adjusted to 4.5. Econazole is detected at 220 nm with a detection limit of 40 ng mL^{-1}.

Sulconazole in plasma is analysed with an ODS column using a mobile phase of acetonitrile–0.01 mol L^{-1} phosphate buffer (pH 8, 66 : 34). Sulconazole is detected at 229 nm with a detection limit of less than 0.5 μg mL^{-1}.

Some of the azole antifungals are used clinically as the racemates, and the enantio-specific HPLC conditions with a chiral stationary phase, tris(chloromethylphenylcarbamate)s of cellulose, are reported. The mobile phase is *n*-hexane-2-propanol (85 : 15) for separation of enantiomers of enilconazole, econazole, miconazole and ornidazole, and *n*-hexane-2-propanol (9 : 1) for bifonazole enantiomers. A similar

type of cellulosic chiral stationary phase (Chiralcel-OD) with a mobile phase of *n*-hexane-2-propanol (9 : 1) is used for separation of sulconazole enantiomers.

Conclusion

Since there is an enormous volume of information on the separation of antibiotics in the literature, readers should be able to find HPLC conditions for almost any antibiotic of interest. Readers are also encouraged to consult the official compendia for analysis of bulk or formulated drugs. For analysis of biological samples, the samples may be directly injected with a column switching technique instead of employing liquid–liquid or solid-phase extraction. For sensitive detection, drugs may be subjected to pre- or post-column derivatization, especially with a fluorescent chromophore. Diastereomeric derivatization is useful for analysis of chiral drugs. Mass spectrometric (MS) detection is another way to increase sensitivity. Indeed, cephem and macrolide antibiotics are analysed with HPLC-MS to detect minute amount of drugs. For cephem antibiotics, capillary HPLC has been coupled with mass spectrometric detection.

See also: **II / Chromatography: Liquid:** Derivatization; Detectors: Fluorescence Detection; Instrumentation.

Further Reading

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Supercritical Fluid Chromatography

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Introduction

The analysis of antibiotics is of primary importance for drug monitoring in pharmacokinetic and health studies, as well as for the quality control of drug production and of numerous food products. As a consequence, the demand for new methods of determination of antibiotics of very different types is continuously increasing. The main methods employed for these analyses include immunoassays and chromatography, as well as various chemical techniques. Among the chromatographic methods, high performance liquid chromatography (HPLC) is the most commonly used, followed by thin-layer chromatography and gas chromatography (GC), while supercritical fluid chromatography (SFC) is still being introduced to this area of application.

In SFC the mobile phase is a fluid subjected to pressures and temperatures near or above the critical point of that fluid, to enhance and control the mobile-phase solvating power. This fact determines that the mobile-phase properties (e.g. diffusivity, density, viscosity) are intermediate between those of gases and liquids and can be varied and controlled by small changes in the pressure or temperature of the systems. The most common fluid used in SFC is carbon dioxide, which has a critical temperature of 31°C, allowing the separation of thermally labile compounds under mild conditions. In general, antibiotics are compounds with intermediate to high polarity, while